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Publication Office
American Public Health Association
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9223 CHROMOGENIC SUBSTRATE COLIFORM TEST (PROPOSED)*

9223 A. Introduction

The chromogenic substrate coliform test utilizes hydrolyzable chromogenic substrates for the detection of enzymes of coliform bacteria. When the chromogenic technique is used the group is defined as all bacteria possessing the enzyme β-D-galactosidase and capable of cleaving the chromogenic substrate, resulting in release of the chromogen. Unlike lactose fermentation methods that permit growth of many aerobic organisms and eliminate or suppress some noncoliforms with inhibitory chemicals, this technique provides nutrients that are more selective and specific for coliform growth. The test can be used in either a multiple-tube or a presence-absence (single 100-mL sample) format. Production of valid results requires strict adherence to quality control procedures. Quality control guidelines are included in Section 9020.

1. Principle

Chromogenic substrates, such as ortho-nitrophenyl-β-D-galactopyranoside (ONPG), are used to detect the enzyme β-D-galactosidase, which is produced by total coliform bacteria. The β-D-galactosidase enzyme hydrolyzes the substrate and produces a color change, which indicates and substantiates a positive test within 24 to 28 h without additional procedures. Noncoliform bacteria, such as species of the genera *Aeromonas* and *Pseudomonas*, that produce small amounts of the enzyme β-D-galactosidase, are suppressed and will not produce a positive response within 28 h unless more than 10^4 colony-forming units (CFU)/mL (10^6 CFU/100 mL) are present.

2. Applications

The chromogenic substrate coliform test is recommended for the analysis of drinking and clean source water samples. Initially, laboratories planning to use this procedure should conduct parallel tests with one of the standard coliform tests over a period of several months to assess the effectiveness of the test for the specific water type being analyzed and to determine the relative number of positive tests by the two techniques.

Water samples containing humic or other material may be colored. If there is background color, compare inoculated tubes to a control tube containing only water sample. In certain waters, high calcium salt content can cause precipitation but this should not affect the reaction.

Do not use the chromogenic substrate test to verify presumptive coliform cultures or membrane filter colonies, because the substrate may be overloaded by the heavy inoculum of weak β-D-galactosidase-producing noncoliforms, causing false-positive results.

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* Approved by Standard Methods Committee, 1992.

9223 B. Chromogenic Substrate Test

1. Substrate

Formulations are available commercially* in disposable tubes for the multiple-tube procedure or in containers that will hold 100-mL samples for the presence-absence approach. Appropriate preweighed portions of the reagent for mixing and dispensing into multiple tubes for 10-mL test portions or other containers for 100-mL samples also are available. The need for good quality assurance and uniformity requires the use of a commercial substrate reagent. Avoid prolonged exposure of the substrate to direct sunlight.

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* Colilert, Environetics, Branford, Conn., or demonstrably equivalent product.

Chromogenic substrate technology reagent system:

The powdered formula contains the following anhydrous compounds (per liter of prepared substrate):

Ammonium sulfate, $(NH_4)_2SO_4$	5.00 g
Manganese sulfate, $MnSO_4$	0.0005 g
Zinc sulfate, $ZnSO_4$	0.0005 g
Magnesium sulfate, $MgSO_4$	0.10 g
Sodium chloride, NaCl	10.0 g
Calcium chloride, $CaCl_2$	0.05 g
Sodium sulfite, Na_2SO_3	0.04 g
Amphotericin B	0.001 g
o-nitrophenyl-β-D-galactopyranoside	0.50 g
4-methylumbelliferyl-β-D-glucuronide	0.075 g
Solanium†	0.50 g
Hepes buffer	
Sodium salt	5.3 g
Organic acid‡	6.9 g

2. Procedure

a. Multiple-tube procedure: Select the appropriate number of tubes per sample with predispensed media for the multiple-tube test and label. Follow manufacturer's instructions for preparing serial dilutions. Aseptically add 10 mL sample to each tube, cap tightly, and mix vigorously to dissolve. The mixture remains colorless. Some particles may remain undissolved throughout the test; this will not affect test performance.

The procedure also can be performed by adding appropriate amounts of the substrate reagent to the sample, mixing thoroughly, and dispensing into five or ten sterile tubes. Incubate at 35 ± 0.5°C for 24 h.

b. Presence-absence procedure: Aseptically add preweighed enzymatic substrate to 100 mL sample in a sterile, transparent, nonfluorescent borosilicate glass or equivalent bottle or container. Optionally, add 100 mL sample to the enzymatic substrate in a sterile container provided by the manufacturer. Aseptically cap and mix thoroughly to dissolve. Incubate at 35 ± 0.5°C for 24 h.

3. Interpretation

After 24 h incubation, examine tubes or containers for a color change. When the substrate is ortho-nitrophenyl-β-D-galactopyranoside (ONPG) it is hydrolyzed by the bacterial enzyme to yield yellow orthonitrophenol; substrates used in other formulations may yield a different color response. A distinct chromogenic response is a positive reaction for total coliforms. If the color response is not uniform throughout the tube, mix by inversion before reading. Compare each tube against the color comparator available from a commercial source of the substrate. If the color intensity is greater than or equal to that of the comparator, total coliforms are present. Samples are negative for total coliforms if no color is observed. If a chromogenic response is questionable after 24 h, incubate up to an additional 4 h. If the chromogen intensifies, the sample is total-coliform positive; if it does not, the sample is negative.

4. Reporting

If performing an MPN procedure, calculate the MPN value from the number of positive tubes as described in Section 9221D. If using the presence-absence procedure, report results as total coliform present or absent in 100 mL sample.

5. Quality Control

Test each lot of substrate purchased for performance by inoculation with three control bacteria: *Escherichia coli*, a total coliform other than *E. coli* (e.g., *Enterobacter cloacae*), and a noncoliform. Avoid using a heavy inoculum. If *Pseudomonas* is used as the representative noncoliform, select a nonfluorescent species. Incubate these controls at 35 ± 0.5°C for 24 h. Read and record results.

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† Solanium is a proprietary mixture of several chemicals including antibiotics. It is available from Environetics, Branford, Conn.

‡ *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid.